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ORIGINAL ARTICLE

Selection and characterization of a Patagonian *Pichia kudriavzevii* for wine deacidification

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indigenous *Saccharomyces cerevisiae*, malic acid, non*Saccharomyces* diversity, wine aroma, wine yeast.

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Abstract

Aims: The purpose of this study was to select autochthonous yeasts with metabolic ability to degrade L-malic acid for its potential use in young wine deacidification.
Methods and Results: Fifty seven Patagonian non*Saccharomyces* yeast of oenological origin were identified by conventional molecular methods and tested in their capability to grow at the expense of L-malic acid. Only four isolates belonging to *Pichia kudriavzevii* species showed this property, and one of them was selected to continue with the study. This isolate, named as *P. kudriavzevii* ÑNI15, was able to degrade L-malic acid in microvinifications, increasing the pH 0·2–0·3 units with a minimal effect on the acid structure of wine. Additionally, this isolate produced low levels of ethanol, important levels of glycerol ($10{\cdot}41 \pm 0{\cdot}48$ g l^{-1}) and acceptable amounts of acetic acid ($0{\cdot}86 \pm 0{\cdot}13$ g l^{-1}). In addition, it improved the sensorial attributes of wine increasing its fruity aroma.
Conclusions: The selection of yeasts for oenological use among non*Saccharomyces* species led to the finding of a yeast strain with novel and interesting oenological characteristics which could have significant implications in the production of well-balanced and more physicochemical and microbiological stable young wines.
Significance and Impact of the Study: The use of *P. kudriavzevii* ÑNI15 as mixed starter with *S. cerevisiae* would eliminate the cultural and cellar operations undertaken to adjust the musts acidity, therefore improving wine quality and reducing production costs.

Introduction

Wine is a highly complex mixture of compounds which largely define its appearance, aroma, flavour and mouth-feel properties. Among these compounds, organic nonvolatile acids have a direct impact on quality of wine and imbalances in this fraction can affect its physicochemical and sensory properties, mainly mouth-feel (Beelman and Gallander 1979; Ruffner 1982; Henick-Kling 1993; Radler 1993; Gao and Fleet 1995; Gawel *et al.* 2007), as well as altering its microbiological estabilty (Delcourt *et al.* 1995; Pretorius 2000).

L-tartaric and L(-)malic acids are the most important constituents of organic nonvolatile acid fraction in grapes and grape musts, accounting for 90% of the titratable acidity, followed by minor concentrations of citric and lactic acid. Succinic and keto acids are present only in trace amounts in grapes, but their concentration

is higher in wines as a result of the fermentative metabolism of micro-organisms, mainly yeasts, associated with winemaking (Whiting 1976; Fowles 1992; Radler 1993; Swiegers *et al.* 2005). Several factors such as grapevine variety, vineyard agricultural practice, temperature, humidity and berry maturity degree, among others, may affect organic nonvolatile acid concentration in grape musts (Ruffner 1982; Flanzy 2000; Volschenk *et al.* 2001). In particular, L(-)malic acid content, directly related to respiratory quotient of berries, is higher in grape musts from cooler regions than those from warmer regions (Ribéreau-Gayon *et al.* 2006). In the Comahue region, located in the Argentinean North Patagonia, which is one of the southernmost winegrowing regions of the world, malic acid concentrations account for 56% of red grape must titratable acidity, reaching 66% in Pinot noir (Caballero *et al.* 2005), the emblematic regional vine variety (Weizman 2009). Additionally to its contribution to wine acidity, malic acid represents a fermentable substrate for other micro-organisms which can spoil the wine before and after bottling (du Toit and Pretorius 2000). Without adjustment of acidity, the wines will be regarded as unbalanced or spoilt (Swiegers *et al.* 2005) hence, malic acid final concentration in wine is of great concern for winemakers and researchers.

The 'wine yeast' *Saccharomyces cerevisiae* does not degrade efficiently malic acid because of the absence of a malate permease (Van Vuuren *et al.* 1995) and the high Km value of its malic enzyme for this substrate (Fuck *et al.* 1973; Kuczynski and Radler 1982; Boles *et al.* 1998). As a consequence, wine L-malic acid has been historically metabolized through malolactic fermentation (MLF), that is the conversion of L-malic to L-lactic acid and carbon dioxide performed by lactic acid bacteria (LAB; Lonvaud-Funel 1999; Muñoz *et al.* 2005). However, spontaneous MLF is a very difficult and unpredictable process in winemaking (Wibowo *et al.* 1985; Thornton and Rodriguez 1996), and the use of commercial starters to induce and guide the process is not always effective (Coucheney *et al.* 2005). Thus, non*Saccharomyces* yeast species belonging to *Schizosaccharomyces* (Viljoen *et al.* 1994, 1999; Thornton and Rodríguez 1996), *Zygosaccharomyces* (Baranowski and Radler 1984) and *Pichia (Issatchenkia)* genera (Okuma *et al.* 1986; Clemente-Jimenez *et al.* 2004; Seo *et al.* 2007; Hong *et al.* 2010) or engineered *S. cerevisiae* strains coexpressing yeast malate permease together with either yeast (Volschenk *et al.* 1997, 2001) or LAB malic enzyme genes (Ansanay *et al.* 1993; Bauer *et al.* 2005; Husnik *et al.* 2006), have been investigated as alternatives to MLF for malic acid degradation during winemaking. Unlike natural strains, engineered 'wine yeast' strains are able to degrade all malic acid present in musts without off flavour production (Pretorius and Høj 2005), yet their use in industrial winemaking has so far been delayed because of consumer anti-GMO aversion (Swiegers *et al.* 2005).

In this work, Patagonian indigenous non*Saccharomyces* yeasts of oenological origin were screened in their capabilities to degradate L(-)malic acid as sole carbon source. Four isolates, identified as *Pichia kudriavzevii* (formerly *Issatchenkia orientalis*), were positive for this test and one of them proved its potential to be used in winemaking.

Materials and methods

Yeasts

Wild yeasts were obtained from North Patagonian spontaneous red winemaking carried out either at industrial scale (10 000 l) in regional cellars named as C, F, N, Ñ and S (Table 1) or at pilot scale (200 l) in an experimental cellar of the Instituto Nacional de Tecnología Agropecuaria, Estación Experimental Agropecuaria (INTA EEA) Alto Valle (noted as I, Table 1), during 2005–2008 vintages. Malbec, Merlot or Pinot noir Samples from initial (12–14 Baumé), middle (six Baumé) and end (<1 Baumé) fermenting musts were appropriately diluted (10^{-3}–10^{-7}) and aliquots of these were spread onto YEPD agar (g l^{-1}: yeast extract 10, glucose 20, peptone 20 and agar 20, pH 4·5) supplemented with 100 ppm of ampiciline (Sigma, Steinheim, Germany). Plates were incubated at 28°C for 2–3 days and isolated colonies were sticked from plates containing between 30 and 300 colony-forming units (CFU) according to their macroscopic features and frequencies to be re-isolated on agar YEPD. Yeast isolates were preserved on YEPD-agar slants, stored at 4°C and subcultured every 2 months. The cultures were also kept at −20°C with 20% v/v glycerol as a cryoprotectant agent.

Yeast identification

Wild yeast identification was performed by conventional methods (Kurtzman and Fell 1998) and by PCR-RFLP analysis of the ITS1-5·8S-ITS2 region from the nuclear rDNA gene complex (Esteve-Zarzoso *et al.* 1999). Gene region amplifications were carried out in a Progene thermocycler (Techne, Cambridge, UK) using ITS1 (5′-TCC GTAGGTGAACCTGCGG-3′) and ITS4 (5′-TCCTCCGC TTATTGATATGC-3′) primers already described (White *et al.* 1990). PCR conditions were as indicated by Esteve-Zarzoso *et al.* (1999). Amplified DNAs (0.5–10 μg) were digested without further purification with *Cfo*I, *Hae*III and *Hinf*I restriction endonucleases (Roche Molecular Biochemicals, Mannheim, Germany) according to the

Table 1 5·8S ITS PCR/RFLP patterns of non*Saccharomyces* yeasts associated with spontaneous alcoholic fermentations from Patagonian wines

		Restriction fragments (bp)				Source		
Species	Amp†	*Cfo* I	Hae III	*Hinf* I	Isolates number	Fermentation stage	Must type	Cellar and vintage
Aureobasidium pullulans[(1)]	600	190 + 180 + 100	450 + 150	290 + 180 + 130	2	Initial	Malbec	ÑN 2006
Candida stellata[(2)]	475	215 + 110 + 80 + 60	475	235 + 235	10	Initial	Merlot	ÑN 2005
					2	Middle		
Clavispora lusitaniae‡	380	210 + 80 + 80	370	190 + 190	5	Initial	Malbec	ÑN 2006
					1*	Middle		
Dekkera anómala[(3)]	800	340 + 340 + 120	800	360 + 190 + 160 + 80	1	Initial	Merlot	ÑN 2005
Hanseniaspora uvarum/ Kloeckera apiculata[(1)]	750	320 + 310 + 105	750	350 + 200 + 180	1	Initial	Merlot	F 2005
					3	Initial	Merlot	S 2005
					1	Initial	Merlot	N 2005
					9	Initial	Malbec	ÑN 2006
					4	Initial	Merlot	C 2007
					4	Initial	Pinot noir	C 2008
					8	Initial	Pinot noir	I 2009
Pichia kudriavzevii/ Candida krusei‡	500	210 + 180 + 70 + 50	400 + 100	220 + 170 + 150	1*	Initial	Malbec	ÑN 2006
					1	Initial	Pinot noir	C 2008
					2	Initial and end	Pinot noir	I 2009
Rhodotorula mucilaginosa‡	600	300 + 230	400 + 120	350 + 220	1*	Initial	Merlot	ÑN 2005
Torulaspora delbrueckii/ Candida colliculosa[(3)]	800	330 + 220 + 150 + 100	800	410 + 380	1	Initial	Malbec	ÑN 2005

[(1)]Sabate *et al.* 2002; [(2)]Hierro *et al.* 2006; [(3)]Esteve-Zarzoso *et al.*1999; *identity confirmed by sequencing of 26S rRNAgene D1/D2 domains; †Amplicon (bp); ‡this work.

supplier's instructions. PCR products and their restriction fragments were separated on 1·5% (w/v) and 3% (w/v) agarose gels, respectively, in TAE buffer (45 mmol l^{-1} Tris-borate, 1 mmol l^{-1} EDTA, pH 8). Gels were stained with ethidium bromide (5 μg ml^{-1}) and visualized under UV light. A 100-bp DNA ladder marker (Gibco BRL, Gaithersburg, MD) served as size standard.

Additionally, the D1/D2 domains of the 26S rRNA gene of the selected isolated were sequenced using NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGACGG-3′) primers (Kurtzman and Robnett 1998). Amplified fragments were then purified using the Perfectprep gel cleanup kit (Eppendorf, Hamburg, Germany) and sequenced. Sequences of the D1/D2 26S rRNA genes were edited and assembled using MEGA ver. 3.1 software and then subjected to a GenBank BLASTN search to retrieve sequences of closely related taxa.

Malic acid assays

Screening. The ability of yeast isolates to use extracellular L-malic acid as carbon and energy source was assayed using MI broth (g l^{-1}: yeast nitrogen base with amino acids 1·7, $(NH_4)_2SO_4$ 5, L-malic acid 20 and bromocresol green 0·1, pH: 3·3; Osothsilp and Subden 1986 slightly modified). Bacteriological tubes containing 5 ml of this medium were inoculated with yeast young culture at a final density of 10^5 cells ml^{-1} and incubated at 25°C under aerobic (shaking at 120 rev min^{-1}) conditions. The presence of malic acid degrading yeasts was visualized by a colour change of green to blue in the medium. Assays using MGI broth (MI broth plus glucose 20 g l^{-1}, pH 3·3) and GI broth (g l^{-1}: yeast nitrogen base with amino acid 1·7, $(NH_4)_2SO_4$ 5, D-glucose 20 and bromocresol green 0·1, pH 3·3) and carried out under the same conditions were used as controls.

In all cases, assays using *S. cerevisiae* ÑIF8, an indigenous yeast strain belonging to a Patagonian cellar, were performed as a comparison.

Growth on malic acid and glucose-malic acid broths. Young cultures of *Pichia kudriavzevii* yeast strain grown on YEPD were inoculated in 200 ml YNB-malic acid broth MB: (g l^{-1}: yeast nitrogen base 17, L- malic acid 20) or YNB- glucose-malic acid broth MGB: (g l^{-1}: yeast nitrogen base 17, glucose 20, L- malic acid 20). Control assays using glucose as single carbon source were also carried out, GB: (20 g l^{-1}). Cultures were maintained at 25°C under aerobic (shaking at 150 rev min^{-1}) and anaerobic conditions, sampled routinely and yeast growth was analyzed using the viable cell counting method. For this purpose, aliquots or appropriate dilutions of culture samples were plated on YEPD plates, incubated at 25°C for 24–48 h and colonies counted. At the end of the assays, yeasts were racked and media pH and composition were analyzed.

In all cases, assays using *S. cerevisiae* ÑIF8 were performed as comparison.

Microvinification

Chemically defined grape juice with similar nitrogen and acidic fraction composition to Patagonian Pinot noir juice (g l^{-1}: glucose 100, fructose 100, potasium tartrate 5, L-malic acid 3, citric acid 0·2, easily assimilable nitrogen 0.208; pH: 3.5; Henschke and Jiranek 1993, modified) was used for microvinification studies with indigenous *P. kudriavzevii* and *S. cerevisiae* ÑIF8. Each yeast strain was plated on YEPD-agar plates and a single colony was picked up, inoculated in 50 ml of YEPD broth (g l^{-1}: yeast extract 10, peptona 20, D(+) glucose 20) and incubated at 25°C for 2 days with agitation (160 rev min^{-1} in a Rolco shaker). Afterwards, yeast cells were collected by centrifugation at 8000 ***g*** for 10 min at 4°C using a Sorvall RC 5C centrifuge. Yeast pellets were washed twice with cold sterile water and resuspended in 5 ml of each must to be counted in a Neubauer chamber.

The fermentations were carried out at laboratory scale, in 250 ml Erlenmeyers containing 200 ml of sterilized synthetic must and inoculated at a final concentration of 10^6 cell ml^{-1}. They were plugged with glass fermentation traps containing sulphuric acid to allow only CO_2 to evolve from the system. Fermentations were carried out at 25°C and their evolutions were determined by weighting. Yeast growth was evaluated by monitoring viable yeast count, determined by plating on YEPD medium using the successive dilution method. Plates were incubated at 28°C for 48 h. On plate, yeast colonies belonging to *P. kudriavzevii* and *S. cerevisiae* were easily distinguished from each other for their significantly different macroscopic features. All fermentations were carried out in triplicate.

Chemical analysis

Organic acid content in the microvinifications' culture media was analyzed by HPLC in a Shimadzu LC-9A liquid chromatograph (Shimadzu, Kyoto, Japan), equipped with a C-18 column and UV detection. A solution of K_2HPO_4 0·2 mol l^{-1}, pH 2·5 was used as mobile phase with a flux of 0·7 ml min^{-1}. The oven was programmed at 40°C for 20 min. Ethanol was determined by headspace gas chromatography in an Agilent 6890N gas chromatograph (Agilent Technologies, Palo Alto, CA),

equipped with a flame ionization detector (FID) and a DB-Alc2 capillary column (30 m × 1·20 μm). Samples were incubated at 40°C and 250 rev min^{-1} for 5 min, and 250 μl of the headspace was injected (syringe temperature 40°C, split injection mode) using an automatic injector CombiPal Agilent G6500. Nitrogen was used as a carrier with a 1·6 ml min^{-1} flow rate and terbutanol was used as an internal standard. The injector temperature was 250°C and column temperature was 40°C for 4 min, then increased to 120°C in a 20°C min^{-1} rate ramp.

Glucose, fructose, L(-)malic acid, L(-)lactic acid and glycerol content were assessed by enzymatic detection kits (Megazyme International Ireland Ltd., Bray Business Park, Bray,Co. Wicklow, Ireland), and pH was measured with a pH 510 Benchtop meter (OAKTON Instruments, Vernon Hills, IL).

Titratable and volatile acidity (TitA and VA) were determined according to published standard methods (Amerine and Ough 1980). Briefly, for TitA determination, 10 ml of the juice or fermented product were added in 90 ml of distilled water, and an alcoholic solution of phenolphthalein was added. The solution was titrated with a 0·1023 N sodium hydroxide solution. VA was removed from the samples by boiling and collected by steam distillation, followed by a titration with NaOH.

The volatile components of the fermented samples were analyzed by the Special Analytical Standards service of the National Wine Institute (Mendoza, Argentina) according to the following protocol: 100 ml of wine were added with 20 μl of R-2-octanol as an internal standard, and volatiles were extracted by means of solid–liquid extraction using an Amberlite XAD-2 polymeric adsorbent and anazeotropic mixture of pentane-dichloromethane solvents (2 : 1). An essential oil drop of the organic fraction was obtained from a Kuderna-Danish concentrator. The profile of the volatile fraction was analysed by injecting 1 μl of the concentrate (split injection mode) in an HP-6890 gas chromatograph (Hewlett–Packard, Wilmington, DE), equipped with a FID and a HP-INNOWax capillary column (50 m × 0·22 mm i.d., 0·25-mm-film thickness). Nitrogen was used as a carrier with a 30 ml min^{-1} flow rate with a column head pressure of 15 psi. The injector and detector temperature were 310 and 350°C, respectively, and the air flow rate was 400 ml min^{-1}. The oven was programmed at 45°C for 5 min, then increased to 165°C in a 2°C min^{-1} rate first ramp, to 280°C in a 10°C min^{-1} rate second ramp and finally kept constant for 10 min. Results were expressed in mg l^{-1}.

Sensorial analysis

Sensorial evaluation of the synthetic wine was performed at 20 ± 2°C by a panel of six judges from INTI (Instituto Nacional de Tecnología Industrial) according to IRAM normatives 20005, 20006 and 20012. Aroma intensity was evaluated using a category scale of five

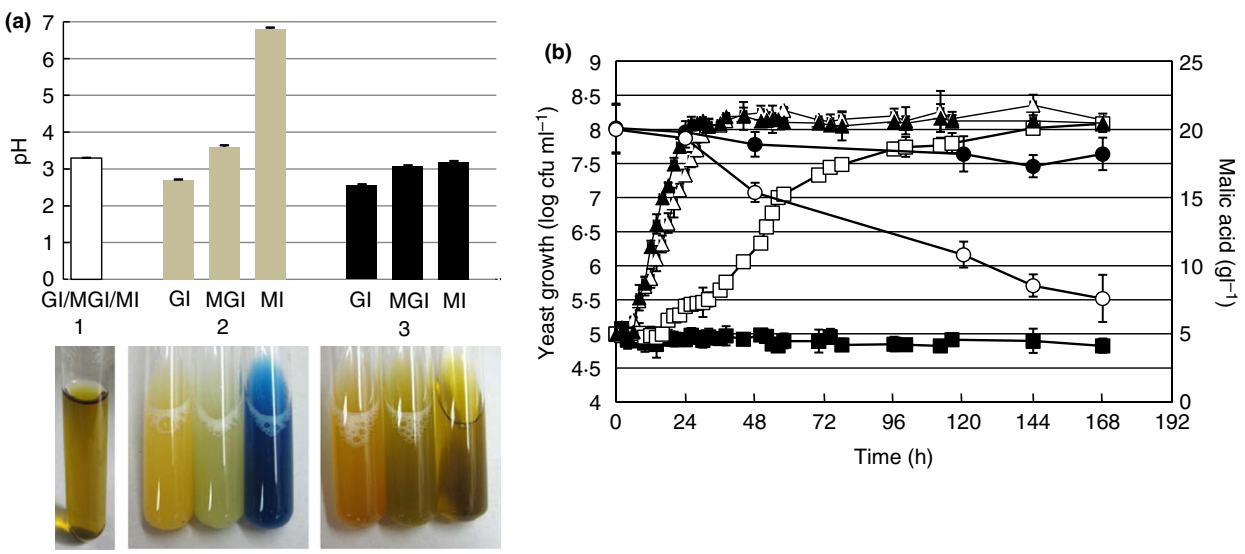

Figure 1 (a) Malic acid degrading yeast screening test. Upper pannel shows pH values for media at 240 h. GI: glucose medium. MGI: glucose + malic acid medium. MI: malic acid medium. (1) Initial condition. (2) *Pichia kudriavzevii* ÑNI15. (3) *Saccharomyces cerevisiae* ÑIF8. Lower pannel: culture media aspect in each condition previously described. (b) Yeast growth (log CFU ml^{-1}) for *P. kudriavzevii* (white symbols) and *S. cerevisiae* (black symbols) in culture media supplemented with malic acid (MB, squares) and glucose (GB, triangles) as carbon sources. Remaining malic acid is also represented (circles).

Table 2 Physiological and biochemical characteristics of Patagonian *Pichia kudriavzevii* isolates

Assimilation tests						Fermentation test					Other tests				
Glucose	Sucrose	D-mannitol	Sorbitol*	D-glucosamine	Succinic acid	Citric acid	DL-lactic acid	DL and L- malic acid	Glucose	Fructose	L-malic acid	Growth at 37°C	Growth at 40°C	Growth in vitamin-free medium	L-lysine assimilation	Cycloheximide resistance (10 mg l^{-1})	Protease activity†
+	−	−	−	+	+	+	+	+	+	+	+	+	+	+	+	−	+

*D-glucitol.
†Either casein or bovine serum albumin tests.

points (0 = none, 5 = extreme) anchored at different points with the corresponding references. Samples were evaluated monadically at random order and judges were instructed to rinse with water extensively and thoroughly between samples. A 10 min rest between samples was recommended to avoid fatigue. Each wine was judged in duplicate.

Statistical analysis

Data were expressed as mean values ± SD (n = number cases). ANOVA for multiple data comparison and Tukey honest significant difference (HSD) *post hoc* tests ($\alpha = 0{\cdot}05$) were performed for mean comparisons. Data normality and variance homogeneity of the residuals were verified by Lilliefors and Bartlett tests, respectively.

Results

Yeasts identification and malic acid screening

Fifty seven wild wine yeasts isolated from eight Patagonian spontaneous red must fermentations and belonging to eight non*Saccharomyces* species (Table 1) were screened for their ability to use L-malic acid as a sole carbon source. For comparative purposes, an indigenous Patagonian, *Saccharomyces cerevisiae* strain with appropriate oenological behaviour (named ÑIF8), was also evaluated.

Of all yeasts assayed, only four isolates named ÑNI15, CNI308, INI3 and INI9 were positive for L-malic acid test, as shown in Fig. 1 for ÑNI15, and they were initially identified as presumably belonging to *Pichia kudriavzevii* (ex *Issatchenkia orientalis*)/*Candida krusei* species based on molecular results (Table 1) along with results from conventional methods (Table 2). The ITS PCR/RFLP restriction pattern observed for this species was similar than that reported by Granchi *et al.* 1999; Clemente-Jimenez *et al.* 2004 and Hierro *et al.* 2006; although some minor differences in band size for the amplified product and for the digested fragments were evidenced. However, all isolates showed the vegetative cell morphology (data not shown) and the biochemical behaviour (Table 2) consistent with those described by Kurtzman and Fell (1998) for *P. kudriavzevii/C.krusei*. As a whole, all isolates showed acidophilic character, temperature tolerance and cicloheximide sensitivity (Table 2). To our knowledge, this is the first report on cicloheximide (actidione) sensitivity for *P. kudriavzevii*. Sequencing of ÑNI15 D1/D2 26S rDNA domains and the ability to sporulate (data not shown) confirmed its identity as *P. kudriavzevii* (Kurtzmann *et al.* 2008). This strain was then selected to continue with the study.

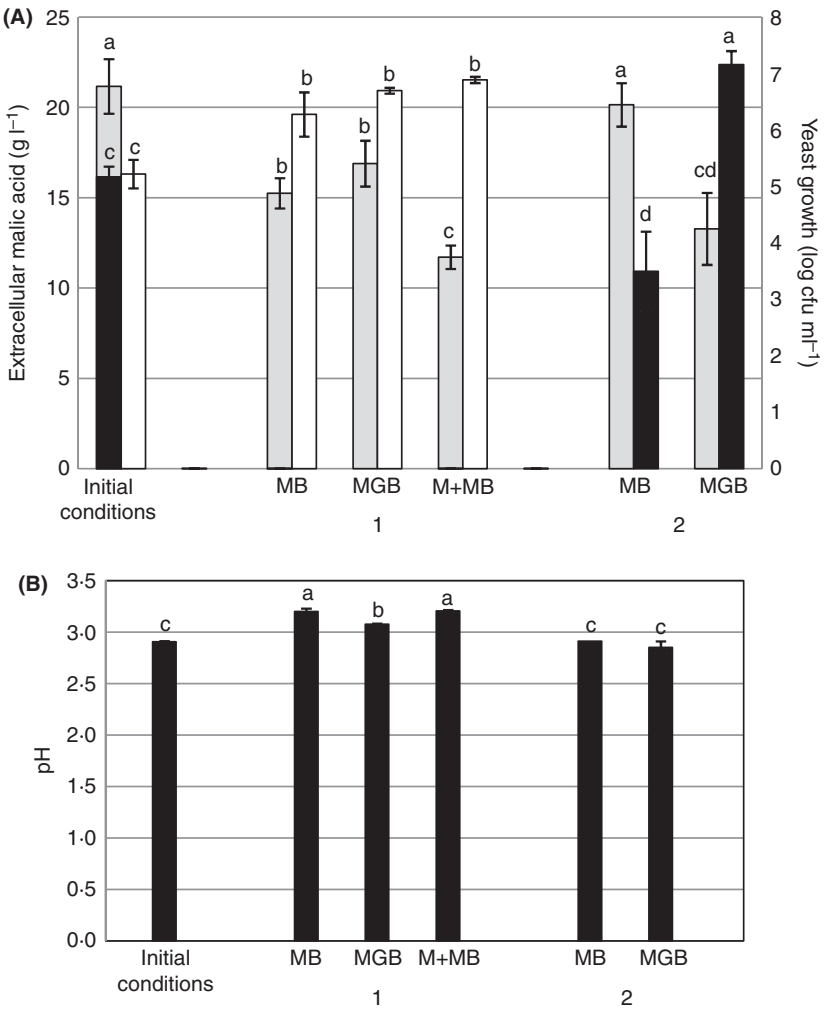

Figure 2 Malic acid broth assays under anaerobic conditions. (A) Remaining extracellular malic acid content (g l^{-1}, grey bars) and yeast growth (log CFU ml^{-1}, white and black bars) for *Pichia kudriavzevii* (1) and *Saccharomyces cerevisiae* (2) cultured in different broths during 7 days. MB: malic acid medium, MGB: glucose + malic acid medium and M + MB: pre-incubation of *P. kiudriavzevii* to 2% L-malic acid during 170 h previous to MB broth assay. (B) pH values obtained from the final broths. Columns displaying different letters within each assay represent significant diferences (ANOVA and Tukey HSD test $n = 2$, $P < 0{\cdot}05$).

Influence of medium conditions on yeast malic acid utilization and growth

Figure 1b shows yeast growth and substrate consumption during L-malic acid broth assays carried out under aerobic conditions. Only *P. kudriavzevii* NÑI15 was able to grow with L-malic acid as a sole carbon source. The maximal population achieved by *P. kudriavzevii* in this medium was as high as the observed in glucose broth (c.a. $1{\cdot}2 \times 10^8$ CFU ml^{-1}) although its growth rate (evidenced by the slopes of the growth curves) was lower in malic acid (Fig. 1b).

Regarding substrate consumption, *P. kudriavzevii* degraded approx. 23% of the L-malic acid within the two first days, reaching 62.3% at the seventh day (Fig. 1b).

Factors such as oxygen and glucose availability influenced the non*Saccharomyces* yeast behaviour related to L-malic acid consumption. For the same time period but under anaerobic conditions, *P. kudriavzevii* showed a lower biomass increase (one logarithmic cycle) and lower L-malic acid utilization (26%; Fig. 2A 1) than those observed in L-malic broth assays under aerobic incubation (three logarithmic cycles and 62.3%, respectively; Fig. 1b). On the other hand, the ability of *P. kudriavzevii* to grow in L-malic acid as single carbon source under anaerobic conditions was similar to that observed in glucose broth (Table 3). Nevertheless, its metabolic behaviour was totally different (Table 3). Fermentation of L-malic acid as single carbon source occurred without ethanol production, yielding acetic and lactic acid as main products and increasing pH from $2{\cdot}90 \pm 0{\cdot}02$ to $3{\cdot}10 \pm 0{\cdot}02$. (Table 3 and Fig. 2B 1). The presence of glucose in the assay medium decreased the ability of *P. kudriavzevii* to degrade L-malic acid but this effect was not significant under anaerobic conditions (Fig. 2A 1). Finally, pre-adapting *P. kiudriavzevii* to 2% L-malic acid during 170 h previous to MI broth assay significantly increased its ability to consume this substrate (45%;

Table 3 Biomass production and physico-chemical characteristics of malic acid (MB) and glucose broths (MG) fermented under anaerobic conditions by *Pichia kudriavzevii* (assay end point: 7 days)

	Malic acid broth		Glucose broth	
Compound (g l^{-1})	Unfermented medium	Fermented medium	Unfermented medium	Fermented medium
Glucose	–	–	20·00 ± 1·40	10·75 ± 3·52
L(-)Malic acid	20·10 ± 1·08	14·69 ± 1·25	–	nd
Citric acid	–	nd		nd
Fumaric acid	–	0·045 ± 0·015		nd
Lactic acid	–	2·200 ± 0·145		1·000 ± 0·161
Acetic acid	–	4·500 ± 0·255		0·700 ± 0·145
Glycerol	–	0·205 ± 0·123		0·920 ± 0·104
Ethanol	–	0·002 ± 0·004		2·660 ± 1·210
pH	2·91 ± 0·01[b]	3·10 ± 0·02[a]	2·90 ± 0·01[b]	2·87 ± 0·02[b]
Biomass (log CFU ml^{-1})	5·17 ± 0·18[a]	6·52 ± 0·61[b]	5·04 ± 0·18[a]	6·37 ± 0·28[b]

Values displaying different superscript letter within the horizontal line are significantly different (ANOVA and Tukey HSD test $n = 2$, $P < 0·05$). nd = nondetected.

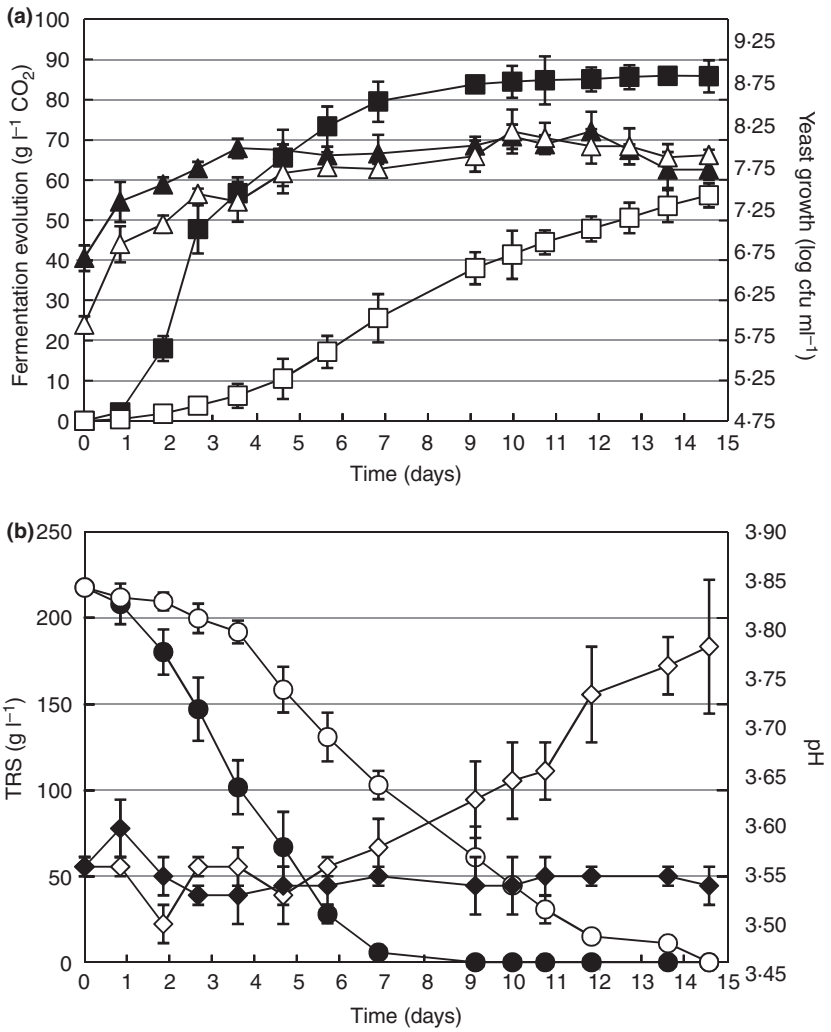

Figure 3 Microvinification analysis in synthetic must. (a) Fermentation evolution (g l^{-1} CO_2, squares) and yeast growth (log CFU ml^{-1}, triangles) along 14 days of fermentation for *Pichia kudriavzevii* (white symbols) and *Saccharomyces cerevisiae* (black symbols). (b) Total Residual Sugars (TRS; g l^{-1}, circles) and pH values (diamonds) evaluated during the analysis.

Table 4 Physicochemical characteristics of wines obtained from synthetic must vinifications carried out by Patagonian *Pichia kudriavzevii* ÑNI15 and *Saccharomyces cerevisiae* ÑIF8 strains at laboratory scale

Parameters	Must	Wines	
		P. kudriavzevii	*S. cerevisiae*
TRS (g l^{-1})*	218·23 ± 1·04	nd	nd
pH	3·55 ± 0·01[b]	3·73 ± 0·07[a]	3·55 ± 0·01[b]
Total acidity (g l^{-1})†	5·85 ± 0·68	5·73 ± 0·38	6·22 ± 0·69
Volatile acidity (g l^{-1})‡	nd	0·86 ± 0·13	0·60 ± 0·06
Ethanol (GL)§	nd	7·81 ± 1·37[b]	10·30 ± 1·40[a]
Glycerol (g l^{-1})	nd	10·41 ± 0·48[a]	6·40 ± 0·96[b]
Organic acids (g l^{-1})			
L (-) Malic acid	3·01 ± 0·28[a]	1·87 ± 0·19[c]	2·34 ± 0·05[b]
Citric acid	0·35 ± 0·21	0·30 ± 0·20	0·38 ± 0·18
Lactic acid	nd	0·05 ± 0·07	0·18 ± 0·04
Succinic acid	nd	0·30 ± 0·14[b]	0·60 ± 0·01[a]
Esters (mg l^{-1})			
Ethyl acetate	nd	nd	8·9 ± 2·1
Ethyl propanoate	nd	29·85 ± 2·30[a]	2·86 ± 0.11[b]
Ethyl octanoate	nd	0·24 ± 0·07[a]	0·02 ± 0·05[b]
Ethyl caproate	nd	0·71 ± 0·10	nd
Higher alcohols (mg l^{-1})			
Butanol	nd	nd	4·08 ± 1·15
n-Pentanol	nd	2·17 ± 0·52	nd
2-methylbutanol	nd	0·06 ± 0·04	0·11 ± 0·02
3-methylbutanol	nd	0·07 ± 0·03	0·09 ± 0·01
1-Phenylethanol	nd	0·18 ± 0·01	nd

Values displaying different superscript letter within the horizontal line are significantly different (ANOVA and Tukey HSD test $n = 2$, $P < 0·05$).
nd, nondetected.
*Total Reducing Sugars.
†Expressed as tartaric acid.
‡Expressed as acetic acid.
§Gay Lussac degrees (ml of ethanol in 100 ml of wine).

Fig. 2A 1), and as it happened in the L-acid malic broth assay, medium pH was also significantly increased (Fig. 2B 1). As expected, *S. cerevisiae* was able to degrade L-malic acid only in the presence of glucose under both aerobic (data no shown) and anaerobic conditions (Fig. 2A 2). However, unlike *P. kudriavzevii* cultures, media pH were not increased (Fig. 2B 2).

Microvinification

Microvinification studies were carried out using synthetic must as a substrate, with similar nitrogen and acidic fraction composition to Patagonian Pinot noir juice. Cultures with indigenous *P. kudriavzevii* and *S. cerevisiae* were performed under anaerobic conditions, emulating wine fermentation. Figure 3 shows the results obtained for both yeast strains. An acceptable yield in biomass was observed in both microvinifications (Fig. 3a). Although both fermentations presented similar sugar concentrations at the end of the process, fermentative efficiency (Fig. 3a) as well as sugar consumption rate (Fig. 3b) was higher for *S. cerevisiae* than for *P. kudriavzevii*. A noteworthy fact, in agreement with what was reported in broth assays, is that *P. kudriavzevii* was again able to raise significantly the medium pH with a minimal effect on acid structure of the wine (decrease of titratable acidity observed in *P. kudriavzevii* wine was not significant compared with control, Table 4), whereas in the *S. cerevisiae* culture pH was constant along the fermentation (Fig 3b).

Analysis of media composition showed a higher ability of *P. kudriavzevii* to metabolize L-malic acid (38%) compared with *S. cerevisiae* (22%; Table 4). Although both yeast strains were able to consume all initial hexoses, wine composition also evidenced significant differences in the fermentative behaviour between them (Table 4). Under the assayed conditions, *P. kudriavzevii* was able to produce important amounts of glycerol but it was a weak producer of ethanol when compared with *S. cerevisiae* ÑIF8. Both yeasts produced relatively low amounts of succinic acid and relatively high amounts of acetic acid. However, *S. cerevisiae* produced more succinic acid than *P. kudriavzevii,* which produced more acetic acid than

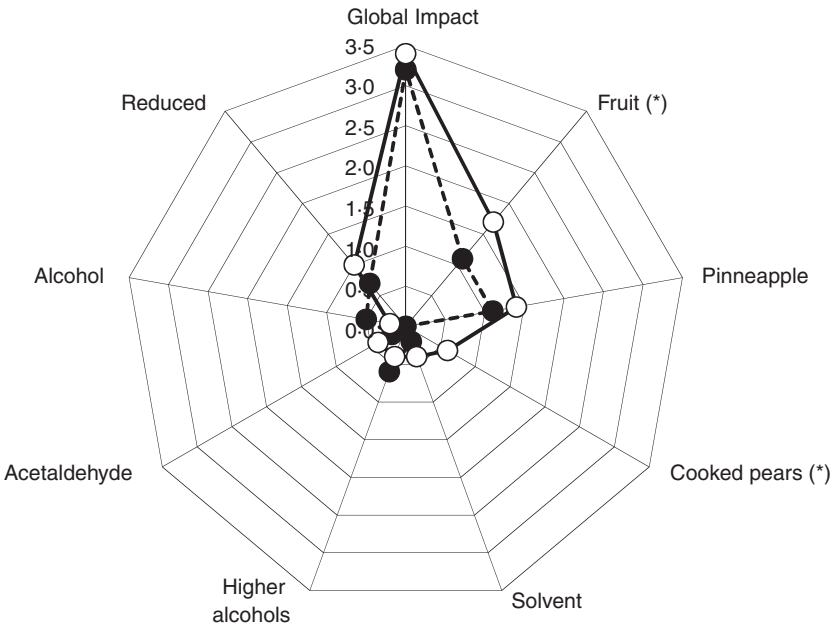

Figure 4 Sensory analysis of wines obtained from laboratory scale fermentations of synthetic must inoculated with *Pichia kudriavzevii* (white circles) or *Saccharomyces cerevisiae* (black circles). ANOVA and Tukey Test, $n = 12$. Asterisks indicate statistical diferences ($P < 0{\cdot}05$).

the former (Table 4). Additionally, both yeasts showed similar ability to produce higher alcohols and esters, but significant differences between their particular profiles were observed (Table 4). *Pichia kudriavzevii* was unable to synthetize ethyl acetate but it showed a good production of ethyl esters from fatty acids when compared with the *Saccharomyces* yeast (Table 4).

Finally, sensorial analysis evidenced significant differences in aromatic perception between *P. kudriavzevii* and *S. cerevisiae* wines. These differences were in favour of the former, which showed a higher fruity and cooked pears aroma than the latter (Fig. 4).

Discussion

Fifty seven indigenous Patagonian yeasts of oenological origin identified as belonging to *Hanseniaspora uvarum*/*Kloeckera apiculata* (53%), *Candida stellata* (21%), *Clavispora lusitaniae* (10%), *Pichia kudriavzevii* (ex *Issatchenkia orientalis*)/*Candida krusei* (7%), *Dekkera anomala* (2%), *Rhodotorula mucilaginosa* (2%), *Torulaspora delbrueckii*/*Candida colliculosa* (2%) and *Aureobasidium pullulans* (1%) species (Table 1) were screened in their abilities to degrade L-malic acid as single carbon source. Only four isolates belonging to *Pichia kudriavzevii* (ex *Issatchenkia orientalis*)/*Candida krusei* were positive for this test and one of them, confirmed in its teleomorphic form, was selected to continue with the study. *Pichia kudriavzevii* is a yeast species often reported in grape musts (Jolly *et al.* 2006; Fleet 2008) but this is the first report of its presence in Patagonian grape musts. Even though this species was detected in a relatively low frequency it was one of the few detected in several musts along with *H. uvarum*, and the only species detected at the final stage of fermentation (Table 1). This result concurrs with what was recently reported for China grape fermentations, where individuals of this species dominated at the end of fermentations (Wang and Liu 2013).

Under the assayed conditions, *P. kudriavzevii* ÑNI15 displayed an extreme tolerance for high L-malate concentrations, levels reaching up to 22 g l^{-1} of L-malate as a sole carbon source. Either in aerobic or anaerobic conditions, it could degrade the compound partially or totally without any negative effect on cell viability and growth (Figs 1b and 2A and Table 3). Yeast species that are recognized for their ability to metabolize extracellular L-malic acid fall into either the Krebs positive or Krebs negative yeast groups (Volschenk *et al.* 2003; Sa-ayman and Viljoen-Bloom 2006). Krebs positive species *Candida utilis*, *Candida sphaerica*, *Hansenula anomala*, *Kluyveromyces lactis* and *Kluyveromyces marxianus* can consume malic acid and other Krebs cycle intermediates as sole carbon and energy source. Krebs negative species *S. cerevisiae*, *Zygosaccharomyces bailii*, *Schizosaccharomyces pombe* and *S. pombe var. malidevorans* can consume malic acid only in the presence of glucose or another assimilable carbon source. The capability of *P. kudriavzevii* ÑNI15 to grow in agar plates with L-malic acid and other Krebs cycle intermediates as a sole carbon source (Table 2) as well as its competence to degrade and grow in L-malic broth assays under aerobic and anaerobic conditions (Figs 1b and 2A, Tables 2 and 3) are consistent with a Krebs positive yeast. Additionally, this ability was induced by the substrate, although, unlike with

what was reported for Krebs positive yeasts (Saayman and Viljoen-Bloom 2006), it was not repressed in the presence of glucose, effect particularlly notable under anaerobic conditions (Fig. 2A 1). This last property constitutes an advantage for the application of *P. kudriavzevii* ÑNI15 in winemaking where high amounts of glucose are present during most of the process. On the other hand, Taillandier and Strehaiano (1991) showed that under anaerobic conditions, *S. pombe* completely metabolized L-malate to ethanol and CO_2. In this pathway, referred as the maloethanolic fermentation, L-malate is decarboxylated to pyruvate by the malic enzyme, with further decarboxylation to acetaldehyde by pyruvate decarboxylase and subsequent reduction to ethanol by alcohol dehydrogenase (Saayman and Viljoen-Bloom 2006). Results showed in Table 3 evidence the absence of maloethanolic fermentation by *P. kudriavzevii* ÑNI15 in L-malic broth assays. While maloethanolic fermentation is a dissimilatory pathway, this result is consistent with the capability observed for this yeast to grow in these assays (Fig. 2a and Table 3). Additionally, significant pH increases in all fermented media were observed (Fig. 2B).

Vinifications of synthetic musts carried out at laboratory scale confirmed the behaviour of *P. kudriavzevii* regarding L-malic acid consumption and the effect observed on pH in broth assays. *Pichia kudriavzevii* ÑNI15 was able to degrade 36% of L-malic acid from de must, increasing significantly its pH in 0·2–0·3 units (Fig. 3b) with minor changes in the acidic structure of wine which is evidenced by the titratable acidity value (Table 4). Similar effects on pH have been observed in raw compost material inoculated with indigenous strains of this species after 2 days cultures under anaerobic conditions (Nakasaki *et al.* 2013).

Acidity adjustment in grape must is an essential step during vinification. In high-acid/low-pH grape musts, typically found in cool-climate regions (pH below 2·9), reduction of TA prior to fermentation is a prerequisite as the onset of alcoholic fermentation by strains of *Saccharomyces* will be negatively affected at such extremely low pH. Viticulturists and winemakers have available several vineyard practices (adequate canopy management, trellising and leafpruning techniques) as well as several cellar operations (skin contact, carbonic maceration, among others) to decrease the acidity of grape musts (Volschenk *et al.* 2006) with a consequent cost in time and money. Additionally, low pH in final wine can be adjusted by blending or, more routinely, by bacterial malolactic fermentation. Although this step is considered the most natural method for wine acidity adjustment, which also contributes to microbial stability and organoleptic complexity, there are a number of pitfalls associated with this biological process (Henick-Kling 1993). In this context, the use of *P. kudriavzevii* ÑNI15 as wine starter would eliminate the cultural and cellar operations undertaken to adjust must acidity, favouring the elaboration of well-balanced, more physicochemical and microbiological stable wines.

Glycerol and acetic acid are the most important by-products of hexose fermentation. When wine glycerol concentration is near 5·2 g l^{-1}, it has a slightly sweet taste leaving an impression of smoothness on the palate (Noble and Bursick 1984) whereas acetic acid concentrations higher than 1·0 g l^{-1} have a negative effect on wine taste and flavour (Swiegers *et al.* 2005). The capability of *P. kudriavzevii* ÑNI15 to produce amounts of glycerol that exceeded this threshold and amounts of acetic acid lower to this threshold level (Table 4) can be considered an advantage for its use in oenology. As glycerol production is largely the result of a stress response, particularly osmoregulation, and redox balance (Hohmann 1997; Remize *et al.* 2003), it is plausible that the amount of glycerol produced by this yeast is related to its response against high osmotic pressure present in musts at initial fermentation stages. In this sense, the use of a *P. kudriavzevii–S. cerevisiae* mixed starter in a sequential form could be an adequate strategy for the production of wines with improved sensorial properties.

Indeed, the must fermented with *P. kudriavzevii* ÑNI15 presented a pleasant 'fruity' aroma which was significantly higher than the one detected in the *S. cerevisiae* wine. A correlation study between volatile fermentation products and sensory descriptors has shown that compounds positively associated with fruit attributes include ethyl propanoate, ethyl octanoate, ethyl dodecanoate, phenylethyl acetate, 2-methylbutanol, 3-methylbutanol and phenylethanol, among others (Torrea *et al.* 2011). The presence of some of these compounds in the *P. kudriavzevii* synthetic wine in concentrations that exceeded their threshold levels and in higher proportions than what was observed in the *S. cerevisiae* wine (Table 4) could explain the sensorial differences between wines (Fig. 4).

Pichia kudriavzevii is one of the yeasts species included in the 2002 IDF inventory, an authoritative lists of micro-organisms with a documented use in food and published as a result of a joint project between the International Dairy Federation (IDF) and the European Food and Feed Cultures Association (EFFCA; Bourdichon *et al.* 2012). Although additional assays using natural grape musts and other fermentation scales must be carried out to confirm the behaviour of *P. kudriavzevii*, the results present in this work position this yeast as a promissory strain with potential application in mixed starters for the production of well-balanced and more physicochemical and microbiological stable young wines.

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Conflict of Interest

No conflict of interest declared.

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